Cytochrome P450 2C9 is involved in flow-dependent vasodilation of peripheral conduit arteries in healthy subjects and in patients with chronic heart failure

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Abstract: BACKGROUND: Flow-mediated dilation (FMD) of human conduit arteries is, in part, related to shear stress-induced release of endothelium-derived nitric oxide (NO). However, NO synthase inhibitors do not completely abolish this FMD-response. Recently, a cytochrome P450 (CYP) epoxygenase of the 2C family was linked to NO- and prostacyclin-independent relaxation of conduit arteries. We therefore evaluated the contribution of CYP 2C9 to FMD in humans. METHODS AND RESULTS: FMD of the radial artery was determined in 12 healthy volunteers by high-resolution ultrasound and analyzed before and after intra-arterial infusion of sulfaphenazole, a specific CYP 2C9 inhibitor, L-NMMA (NO synthase inhibitor) and co-infusion of both. Endothelium-independent vasodilation was characterized after intra-arterial infusion of SNP. FMD was reduced after sulfaphenazole (11.5+/-0.87% vs. 7.4+/-0.95%, $p<0.01$), after L-NMMA (6.0+/-0.71%; $p<0.01$), and after co-infusion 3.9+/-0.73% ($p<0.05$ vs. L-NMMA; $p<0.01$ vs. sulfaphenazole). Sulfaphenazole had no effect on endothelium-independent vasodilation. In patients with chronic heart failure, the portion of FMD blocked by sulfaphenazole was not affected. CYP 2C was detected by immunohistochemistry in radial artery samples obtained from patients undergoing coronary bypass surgery. CONCLUSIONS: FMD in human conductance arteries is reduced after inhibition of CYP 2C9, supporting the concept that CYP 2C metabolites contribute to endothelium-mediated vasodilation of peripheral conduit arteries in vivo. In patients with heart failure, the CYP-dependent FMD appears to be preserved.

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Cytochrome P450 2C9 is involved in flow-dependent vasodilation of peripheral conduit arteries in healthy subjects and in patients with chronic heart failure

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Running title: Cytochrome P450 2C9 and endothelial function

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Abstract:

Background: Flow-mediated dilation (FMD) of human conduit arteries is, in part, related to the shear stress-induced release of endothelium-derived nitric oxide (NO); which is an important regulator of vascular tone. However, although NO synthase inhibitors attenuate FMD in humans they are unable to abolish the response. Recently, a cytochrome P450 (CYP) epoxygenase belonging to the 2C family was linked to the NO- and prostacyclin-independent relaxation of conduit arteries. We therefore evaluated the contribution of CYP 2C9 to FMD in humans.

Methods and results: FMD of the radial artery was determined in 12 healthy volunteers by high resolution ultrasound. FMD was analyzed during control conditions, after intra-arterial infusion (5 min) of the specific inhibitor of CYP 2C9, sulfaphenazole (4 mg/min), L-NMMA (NO-synthase-inhibitor, 7 µmol/min) and after co-infusion of both compounds. Endothelium-independent vasodilation was characterized after intra-arterial infusion of SNP (9 µg/min, 5 min). FMD under control conditions was 11.5±0.87%, after sulfaphenazole 7.4±0.95% ($p<0.01$ vs. control), after L-NMMA 6.0±0.71% (<0.01 vs. control), and after co-infusion 3.9±0.73% ($p<0.01$ vs. control, $p<0.05$ vs. L-NMMA, $p<0.01$ vs. sulfaphenazole). Sulfaphenazole had no effect on endothelium-independent vasodilation (SNP: 19.6±1.87%, SNP + sulfaphenazole: 20.2±2.16, p=n.s.). In patients with chronic heart failure, the portion of FMD blocked by sulfaphenazole was not affected. CYP 2C was detected by immunhistochemistry in radial artery samples obtained from patients undergoing coronary bypass surgery.

Conclusions: FMD in human conductance arteries is reduced after inhibition of CYP 2C9, supporting the concept that CYP 2C metabolites contribute to the endothelium-mediated vasodilation of peripheral conduit arteries in vivo. In patients with heart failure, the CYP-dependent FMD appears to be preserved.

Introduction

Flow-mediated dilation of conductance arteries is a principle mechanism by which changes in flow affect vascular tone and vasodilation. Numerous experimental and, more recently, clinical studies have established that the shear-stress induced release of nitric oxide (NO) contributes to the flow-mediated vasodilation of arterial conductance vessels. However, we and others have noted that a residual response remains after blocking the production of NO and the generation of prostacyclin. [1-3] In fact, prolonged episodes of reactive hyperemia result in enhanced residual FMD that is not affected by NO synthase inhibitors.[3] Experimental studies have demonstrated that additional endothelium-derived factors can contribute to FMD in animals. Indeed, in some arteries an endothelium-derived hyperpolarizing factor (EDHF) has been implicated in this process and it has been suggested that the residual FMD observed after the NO synthase blockade can be attributed to an EDHF-mediated vasorelaxation.[4]

It has previously been demonstrated that a cytochrome P450 epoxygenase of the 2C family (CYP 2C) expressed in the endothelium plays a crucial role in the generation of EDHF-mediated responses in porcine coronary arteries.[5] More recently, CYP 2C9 has been identified in the endothelium of human mammary arteries and has been shown to generate the vasodilator 11,12-epoxyeicosatrienoic acid (11,12-EET).[6] However, in addition to generating EETs, the CYP 2C epoxygenase can also generate oxygen-derived free radicals which attenuate the bioavailability of NO.[6] Notably, this “EDHF synthase” can be efficiently and selectively inhibited by the sulfonamide sulfaphenazole both in animals and humans.[5,7-9]

The aim of the present study was to determine whether CYP 2C9 contributes to the flow-induced dilation of conduit arteries in humans and whether the contribution of the

enzyme to FMD is attenuated or exaggerated in disease states such as chronic heart failure. We therefore studied the effect of sulfaphenazole on FMD of the radial artery in healthy volunteers and patients with chronic heart failure.

Methods

Study population

The study protocol was approved by the Ethics Committee of the Medizinische Hochschule Hannover. Twelve young healthy volunteers, who gave their informed consent, were included and their baseline characteristics are outlined in table 1. All subjects were nonsmokers, normotensive, did not take any cardiac medication, and had normal serum cholesterol levels. Patients with chronic heart failure (NYHA functional class II and III) were also included in this study. The clinical characteristics of patients with chronic heart failure are depicted in table 2. The functional NYHA class was evaluated the day before FMD-measurement.

Measurement of FMD

Radial artery diameters were measured using a high-resolution ultrasound system (ASULAB) with a precision of ± 2.5 µm. This method is well established in our laboratory, has an excellent reproducibility and variability, as reported previously.[1,2] After insertion of a polyethylene catheter in the left brachial artery (nondominant arm), blood flow velocity was recorded continuously by an 8-MHz Doppler probe, and radial artery diameter was determined every 30 seconds until stable baseline values were obtained (30 minutes). To prevent prostaglandin production, 250 mg aspirin (Aspisol®, Bayer AG, Leverkusen, Germany) was given intravenously 30 minutes before measurements, a dose known to effectively inhibit vascular cyclooxygenase activity. Thereafter, a wrist arterial occlusion (8 minutes) distal of the transducer was performed and FMD in response to reactive hyperemic blood flow was assessed. After release of the arterial occlusion, arterial diameter was determined at 20 second intervals for 2 minutes then every 30 seconds until the diameter returned to baseline. Arterial blood pressure and

heart rate were measured on the contralateral arm by the cuff technique. Next, sulfaphenazole (Merck Biosciences AG, Läufelfingen, Switzerland; diluent saline 0.9 %) was infused (4 mg/min for 5 minutes) intra-arterially (brachial artery), followed by intra-arterial saline infusion during arterial occlusion and determination of FMD after release of arterial occlusion. It has been reported previously that administration of a similar dose of sulfaphenazole resulted in local plasma levels, high enough to effectively inhibit CYP 2C acitivity[10]. Next, N-monomethy-L-arginine (L-NMMA, Calbiochem, CN Biosciences, La Jolla, CA, USA; in saline 0.9 %) was infused intra-arterially (brachial artery) to inhibit the endothelial NO synthase (7 µmol/min for 5 minutes), FMD was determined after wrist arterial occlusion for 8 minutes. After again obtaining baseline levels, an intra-arterial (brachial artery) co-infusion of sulfaphenazole and L-NMMA (doses as mentioned above, each 5 min) was performed and FMD was determined. Finally an intra-arterial (brachial artery) infusion of sodium-nitroprusside (SNP; Schwarz Pharma, Monheim, Germany; 9 µg/min for 5 minutes in saline 0.9 %) to assess endothelium-independent vasodilatory capacity was performed.

All measurements were performed in a quiet room with an ambient temperature between 22° and 24° C. Alcohol and caffeine were prohibited within 12 hours of the study.

Immunhistochemistry

Radial artery specimens were obtained from patients who underwent coronary artery bypass graft surgery (n=6). Freshly isolated radial artery specimens were washed in ice cold PBS and immediately embedded in tissue freezing medium (Jung) and stored at -80°C. Frozen sections were fixed in phosphate-buffered formaldehyde solution (4%), permeabilized using Triton X-100 (0.2%) and blocked with bovine serum albumin

solution (3%) and horse serum (5%) in phosphate-buffered saline. CYP 2C was detected using a specific polyclonal CYP 2C antibody (kindly provided by Dr. E. Morgan, Atlanta, GA) and ß-actin using a monoclonal antibody (Sigma). Preparations were then washed and incubated with fluorescent secondary antibodies (Alexa), mounted and images were acquired by laser scanning microscopy (LSM 510 meta, Carl Zeiss, Jena, Germany).

Statistics

Data are expressed as the mean ± SEM and data were analyzed by ANOVA for repeated measures followed by Student Newman Keuls test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Arterial blood pressure and heart rate did not change during the study protocol. The intra-arterial infusion of sulfaphenazole was well tolerated, and no side effects were observed. Radial arterial diameters are depicted in table 3 (healthy subjects) and table 4 (heart failure patients). No changes of the baseline diameter were observed after infusion of sulfaphenazole or L-NMMA.

Effect of sulfaphenazole on FMD

FMD during infusion of saline was 11.5 $\pm$ 0.87 %, FMD during infusion of sulfaphenazole was 7.4 $\pm$ 0.95 %, $p<0.01$. Thus, FMD of the radial artery was significantly reduced during infusion of sulfaphenazole (figure 1). To determine whether an effect of sulfaphenazole was also apparent after inhibition of the NO synthase, FMD was determined during infusion of the NO synthase inhibitor L-NMMA (6.0 $\pm$ 0.78 %) as well as during the co-infusion of both compounds (FMD 3.9 $\pm$ 0.73 %). The co-infusion

resulted in a further significant inhibition of FMD as compared to L-NMMA alone in healthy subjects (figure 1).

Effect of Sulfaphenazole on endothelium-independent vasodilation

To determine whether the effect of sulfaphenazole is specific for endothelium-mediated vasodilation, we assessed the effect of sulfaphenazole on endothelium independent relaxation elicited to sodium nitroprusside (SNP). As shown in figure 2, sulfaphenazole had no effect on the SNP-induced, endothelium-independent vasodilation (FMD 19.6 $\pm$ 1.87 % vs 20.2 $\pm$ 2.16 %, p= n.s).

Effect of drugs infusions on reactive hyperemic blood flow

Blood flow before and after release of the cuff was assessed to ascertain whether or not the stimulus, i.e. the increase in flow, was similar during the different drug infusions. Blood flow at rest was significantly reduced after sulfaphenazole infusion in healthy subjects (table 3), however, neither of the compounds had a significant effect on reactive hyperemic blood flow, suggesting that the stimulus for flow-dependent dilation was similar in all groups. The results for radial artery blood flow are depicted in table 3 for healthy subjects and in table 4 for heart failure patients.

Effect of sulfaphenazole on FMD in patients with heart failure

When compared with responses observed in healthy volunteers, flow-mediated dilation was attenuated in patients with heart failure. Sulfaphenazole significantly reduced FMD in patients with heart failure ($p<0.01$), an effect that was similar to the observations in healthy volunteers. The co-infusion of L-NMMA and sulfaphenazole caused an additional reduction in FMD compared to the infusion of L-NMMA alone. Sulfaphenazole had no effect on the endothelium-independent vasodilation elicited by SNP in patients with heart failure (data not shown). To analyse the effect of sulfaphenazole in severe heart failure patients, subjects were divided in two groups. One group had a FMD lower

than the median (7.76%), the other group had a FMD greater than the median. However, there was still a significant inhibition of FMD after infusion of sulfaphenazole in patients with a FMD below the median value (FMD control 5.7 $\pm$ 0.49 %, FMD after sulfaphenazole infusion 3.4 $\pm$ 0.72 %, $p < 0.05$).

Immunhistochemical detection of CYP 2C in radial arteries

In immunhistochemical studies, the expression of CYP 2C protein was detected in endothelial cells and in some samples in cells infiltrating the medial layer of the radial artery (figure 4), the vessel segment which was investigated in vivo by our echotracking device.

Discussion

The results of the present study demonstrate that FMD in human conductance arteries is substantially reduced after inhibition of CYP 2C9 supporting the concept that a factor generated by this enzyme contributes to flow-mediated, endothelium-dependent vasodilation of peripheral conduit arteries in vivo. In patients with heart failure, the CYP-dependent FMD appears to be preserved. Importantly, the expression of CYP 2C was detected by immunhistochemistry in radial artery samples obtained from patients undergoing coronary bypass surgery, the same vascular bed in which FMD-measurements were performed in the in vivo studies.

Recent studies by our group and others have shown that the dilation of conduit arteries in response to reactive hyperemia is reduced by inhibitors of NO synthesis, suggesting an important role of NO in FMD[1-3]. However, several reports have indicated that FMD is a complex phenomenon involving the synthesis and release of more than one factor and that a substantial portion of the response may be independent of NO production[2,3].

In both animals and humans, coronary FMD in response to prolonged hyperemia has previously been reported to be resistant to NO synthase inhibition.[9,11]

Recently, Archer et al.[6] demonstrated that human left internal mammary arteries express CYP 2C9, generate 11,12-EET and induce the hyperpolarization of vascular smooth muscle cells in response to stimulation. Indeed, based on the fact that NO- and prostacyclin-independent, or EDHF-mediated responses in the mammary arteries studied were sensitive to a CYP epoxygenase inhibitor, a CYP 2C-like EDHF synthase was suggested to account or approximately 40% of the net endothelium-dependent relaxation in vitro. This quantitative estimation of the contribution of EDHF to endothelium-dependent relaxation is consistent with our present *in vivo* observations in human conduit arteries, using increased flow as a stimulus. In agreement with the observations made by Archer et al.[6], who found CYP 2C expression in left internal mammary arteries, we found that CYP 2C is expressed in a human radial artery. Although a sulfaphenazole-sensitive process was previously reported to contribute to the phenomenon of exercise-induced hyperaemia in skeletal muscle of healthy volunteers,[12] three recent studies have reported that CYP 2C does not contribute to the agonist- induced vasorelaxation of human forearm resistance arteries.[9,10,13] The reasons for these contrasting findings are not entirely clear but are most probably related to the differential expression of CYP 2C enzymes in different vascular beds. Indeed, CYP 2C is not homogenously expressed throughout the vasculature and even within the porcine coronary circulation there are marked differences.[14] Thus, it is possible that the apparent lack of effect of sulfaphenazole reported in previous studies can be attributed to the fact that the vasodilations elicited by acetylcholine and bradykinin reflect an integrated response in CYP 2C-expressing as well as CYP 2C-deficient vessels. It cannot be excluded, however, that FMD is a better activator of the

CYP expressed in endothelial cells than the agonists that are generally used to assess vasodilator function. We have observed that CYP 2C is expressed in the human radial artery and sulfaphenazole attenuates the FMD of this artery, indicating that a CYP 2C enzyme contributes to the NO- and prostacyclin-independent (EDHF-mediated) responses in human conduit arteries, rather than in the forearm microcirculation.

In the latter vascular bed however, i.e. in the forearm microcirculation, an increase in the activity of a CYP 2C enzyme has been associated with a markedly different effect and FichtIscherer et al.[9] reported that in patients with manifest coronary artery disease the inhibition of CYP 2C results in a marked improvement of the acetylcholine-stimulated, endothelium-dependent and NO-mediated vasodilations. The latter effect was attributed to the inhibition of CYP 2C-derived reactive oxygen species which would be expected to react with NO and thus decrease its bioavailability.[8,9] Similarly, in patients with hypertension there a pronounced effect of sulfaphenazole on the acetylcholine stimulated endothelium-dependent vasodilation has recently been observed.[13] In the present study we have characterized for the first time the effect of sulfaphenazole on the endothelium-dependent vasodilation in patients with chronic heart failure in a conductance artery. In patients with heart failure, sulfaphenazole had a similar effect on endothelium-dependent vasodilation as compared to healthy subjects, suggesting that the CYP2C response cannot compensate for impaired NO-mediated vasodilation in patients with CHF.

A number of different factors and/or mechanisms have been proposed to underlie the EDHF phenomenon and compensate for the loss of NO by acting as a back up system to maintain FMD (for review see [15]). The literature relating to the identity of putative EDHFs is highly confusing, perhaps more so since - in addition to the release of

hyperpolarizing concentrations of K^+ ions from endothelial cells or a CYP 2C-derived metabolite of arachidonic acid,[15] -endothelium-derived H_2O_2 has also been proposed to act as an EDHF in human and murine arteries.[16-19] Notably, Miura et al. have reported that an H_2O_2-like EDHF contributes more to FMD in patients with coronary artery disease (CAD) than in individuals without CAD.[17] Since a CYP 2C epoxygenase is a functionally significant source of reactive oxygen species within the vascular wall [8] and H_2O_2 may act as an EDHF[16] it is tempting to speculate that a CYP enzyme may underlie both responses.

As NO-mediated responses are attenuated in heart failure and physiologically relevant concentrations of NO attenuate CYP activity in healthy vessels[20,21], we expected the attenuated NO-dependent responses to be associated with an alleviation of the intrinsically inhibited EDHF response. However, rather than recording a more pronounced contribution of EDHF to FMD in patients with chronic heart failure we found that the inhibitory effects of sulfaphenazole on FMD were comparable in normal individuals and in patients with heart failure. At this point it is important to note that in previous studies have shown that NO-dependent FMD is impaired in patients with heart failure.[1,2] However in the present investigation, the extent of blockade of FMD by L-NMMA was somewhat reduced as compared to our earlier studies [1,2]. Our patients were treated with ACE-inhibitors, statins and aldosterone inhibitors which, in contrast to our previous observations[1,2], have all been shown to improve endothelial function, at least partly, by reducing oxidative stress.[22-24] Drug treatment may even increase the expression of CYP 2C as nifedipine[25], cerivastatin and fluvastatin[26] have all been reported to increase CYP 2C expression in endothelial cells and in isolated arteries *in vitro*. Thus it is possible that these compounds also indirectly affected responsiveness

to sulfaphenazole by interfering with the generation of reactive oxygen species and/or the generation and actions of the CYP-derived EDHF/EET´s. Effective treatment of heart failure patients is associated with a reduced oxidative stress and may indeed provide an explanation for the fact, that NO-mediated FMD was not impaired in the patient population studied.

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Figure legends

Figure 1: Change in radial artery diameter (%) during reactive hyperemia (flow-dependent dilation) after wrist occlusion in healthy volunteers under control conditions (saline infusion) and after infusion of sulfaphenazole, L-NMMA and co-infusion of sulfaphenazole and L-NMMA)

Figure 2: Change in radial artery diameter (%) during reactive hyperemia (flow-dependent dilation) after wrist occlusion in patients with chronic heart failure under control conditions (saline infusion) and after infusion of sulfaphenazole, L-NMMA and co-infusion of sulfaphenazole and L-NMMA).

Figure 3: Results of immunhistochemical studies of radial artery specimens (n=6) obtained from patients undergoing coronary bypass surgery. CYP2C is labeled red, β-actin is labeled green and VE-cadherin is labeled blue. The arrows point to the endothelial cells stained for CYP 2C.

Table 1: Baseline characteristics (healthy volunteers, n=12)

Age	24.2 ± 3.3 years
Gender, male	9 (75)
Hypertension	0
Current Smoker	0
Total cholesterol, mg/dl	158.8 ± 10.7
LDL-Cholesterol, mg/dl	103.3 ± 9.8
HDL-Cholesterol, mg/dl	54.4 ± 2.4
CRP, mg/l	3.3 + 0.8

mean ± SEM
Figures in parentheses are percentages

Table 2: Baseline characteristics (CHF patients, n=12)

Age	66.8 ± 2.1 years
Gender, male	11 (92)
Ischemic cardiomyopathy	8 (75)
Dilated cardiomyopathy	4 (25)
NYHA functional class II	3 (25)
NYHA functional class III	9 (75)
Hypertension	8 (75)
Current smoker	0
NT-pro BNP, ng/l	2804 ± 961
CRP, mg/l	17 ± 9
Hemoglobin, g/dl	13.7 ± 0.6
Serum sodium, mmol/l	137 ± 1.2
Serum creatinine, µmol/l	104 ± 12
Total cholesterol, mg/dl	175 ± 20
LDL cholesterol, mg/dl	110 ± 15
HDL cholesterol, mg/dl	43 ± 5
Medical treatment	
ASA	9 (75)
ACE-inhibitor/AT-blocker	12 (100)
ß-blocker	11 (92)
Diuretics	10 (83)
Spironolactone	8 (67)
Statin	8 (67)

mean ± SEM; Figures in parentheses are percentages

Table 3: Radial arterial diameters and radial artery blood flow at baseline and during reactive hyperemia in healthy volunteers: Effect of sulfaphenazole, L-NMMA and co-infusion.

Arterial diameter, mm	**Baseline**	**Reactive hyperemia**	**Absolute change**
Control	2.692 ± 0.10	3.010 ± 0.12	0.317 ± 0.03
Sulfaphenazole infusion	2.692 ± 0.09	2.883 ± 0.11*	0.191 ± 0.03*
L-NMMA infusion	2.707 ± 0.08	2.872 ± 0.09*	0.166 ± 0.02*
Co-infusion	2.725 ± 0.08	2.831 ± 0.09*	0.106 ± 0.02*

Radial artery blood flow, ml/min	**Baseline**	**Reactive hyperemia**
Control	19.79 ± 3.31	43.66 ± 5.86
Sulfaphenazole infusion	15.06 ± 3.40 *	44.57 ± 5.33
L-NMMA infusion	18.78 ± 2.36	44.04 ± 6.05
Co-infusion	15.16 ± 3.78 *	45.18 ± 5.46

mean ± SEM; * $p<0.05$ vs. control infusion

Table 4: Radial arterial diameters and radial artery blood flow at baseline and during reactive hyperemia in heart failure patients: Effect of sulfaphenazole, L-NMMA and co-infusion.

Arterial diameter, mm	**Baseline**	**Reactive hyperemia**	**Absolute change**
Control	3.198 ± 0.13	3.460 ± 0.15	0.262 ± 0.04
Sulfaphenazole infusion	3.245 ± 0.13	3.398 ± 0.13*	0.153 ± 0.02*
L-NMMA infusion	3.233 ± 0.14	3.359 ± 0.14*	0.126 ± 0.02*
Co-infusion	3.265 ± 0.13	3.358 ± 0.13*	0.093 ± 0.01*

Radial artery blood flow, ml/min	**Baseline**	**Reactive hyperemia**
Control	29.97 ± 7.55	60.15 ± 10.09
Sulfaphenazole infusion	27.02 ± 7.23	51.02 ± 7.33
L-NMMA infusion	22.79 ± 4.3	49.82 ± 7.37
Co-infusion	24.84 ± 3.73	50.71 ± 5.46

mean ± SEM; * $p<0.05$ vs. control infusion

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Figure 1: Endothelium-mediated, flow-dependent vasodilation of peripheral conduit arteries in healthy volunteers (n=12).

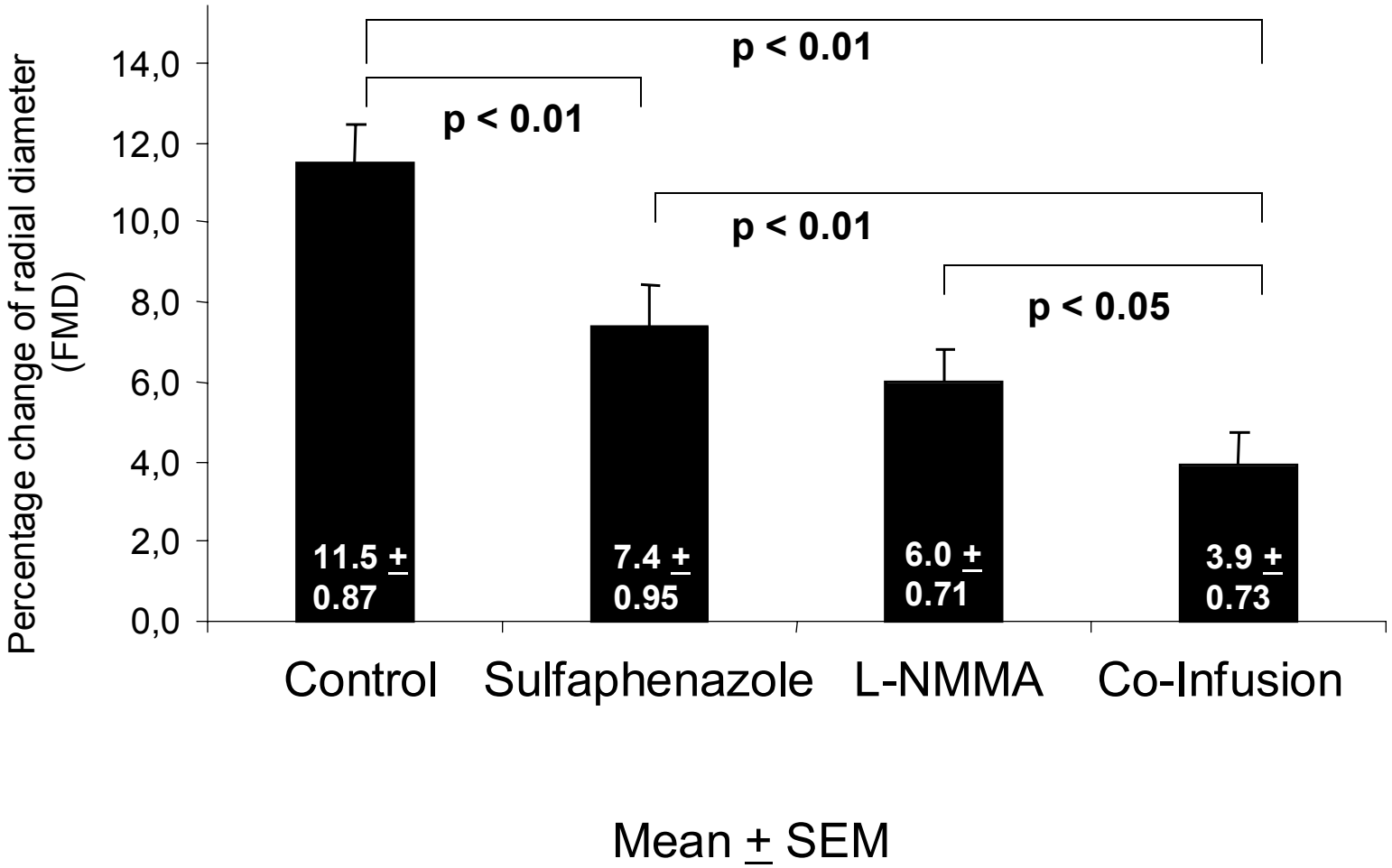


Figure 2: Endothelium-mediated, flow-dependent vasodilation of peripheral conduit arteries in patients with heart failure (n=12).

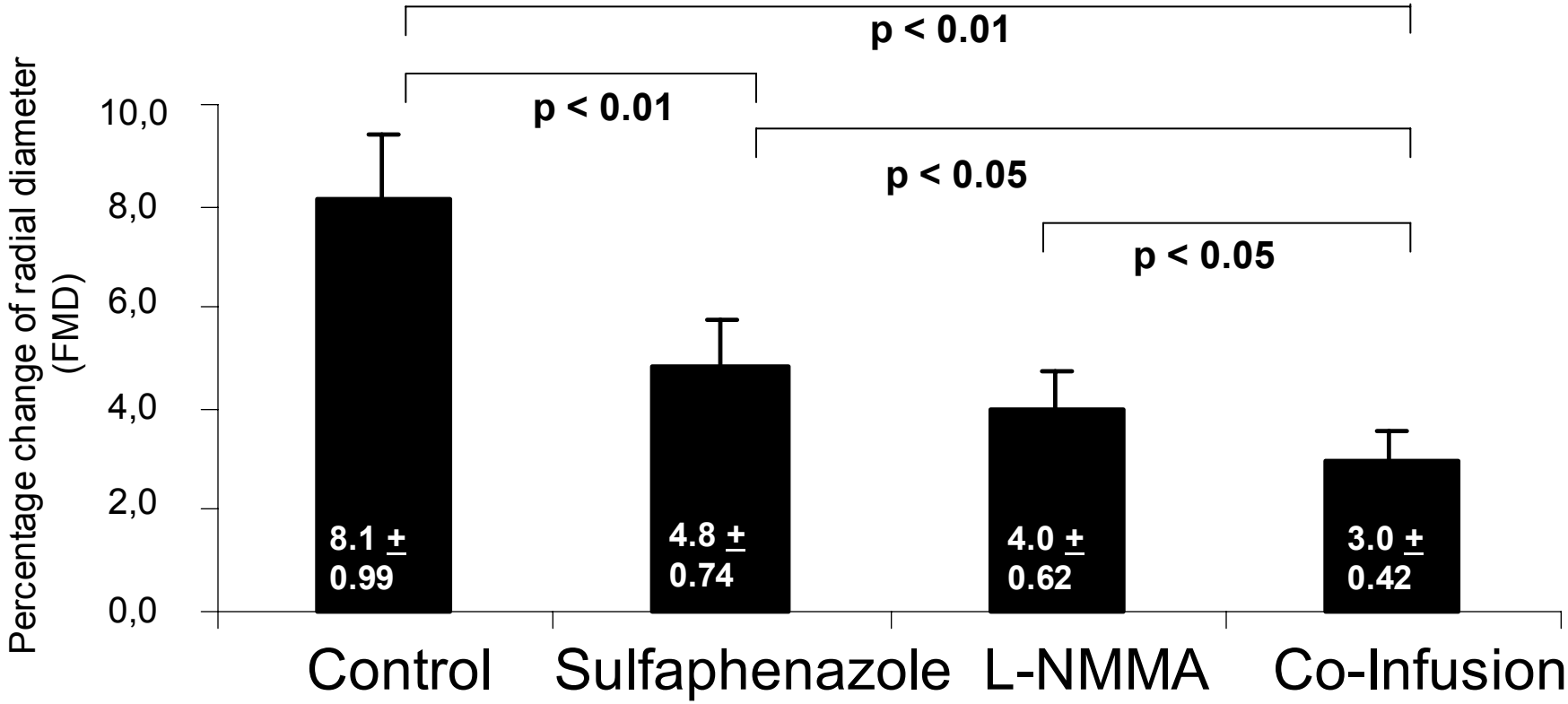
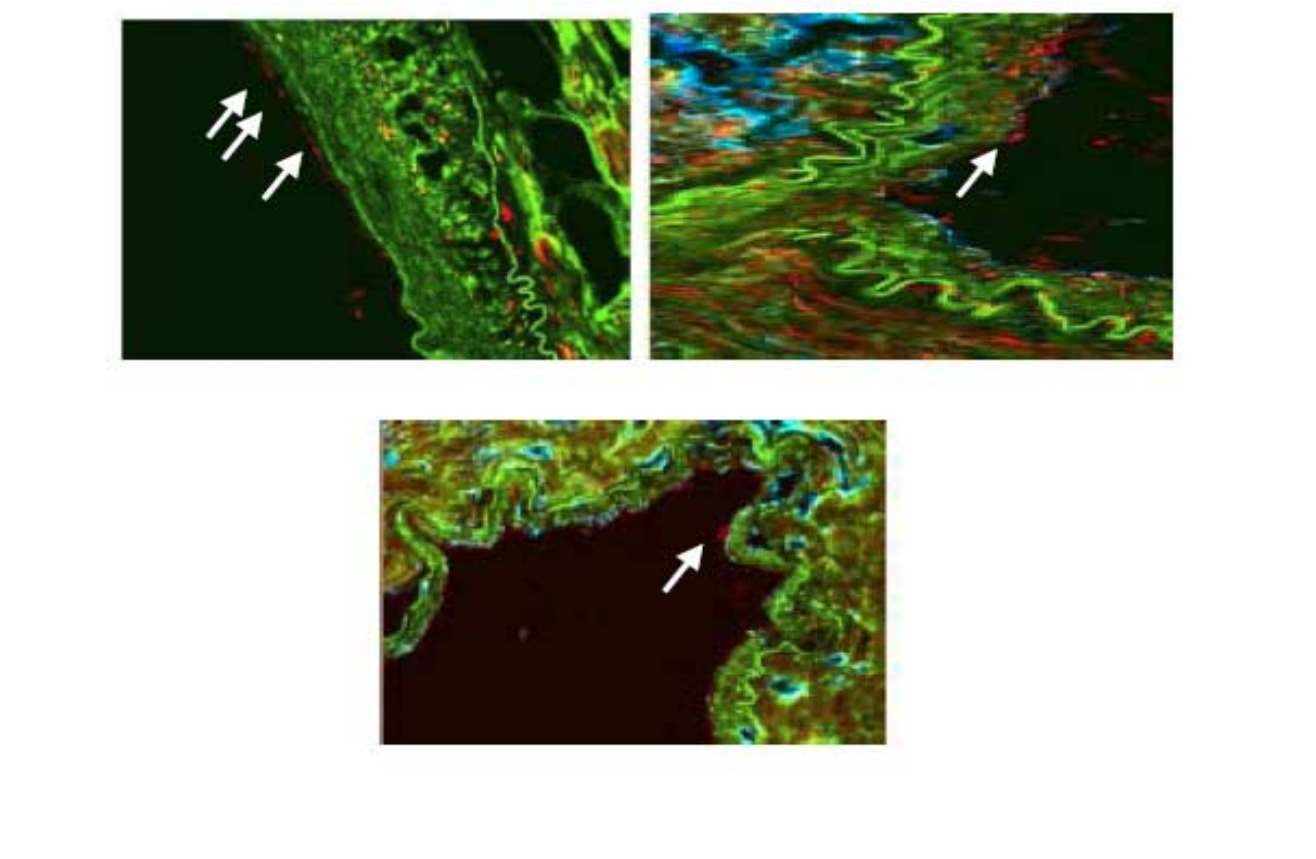


Figure 3:



*** Word Count**

Cytochrome P450 2C9 is involved in flow-dependent vasodilation of peripheral conduit arteries in healthy subjects and in patients with chronic heart failure.

Word count (without tables and references): 3055 words.